L-FORMS OF BACTERIA

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[Following is a translation of the Russian language article by V. D. Timakov and G. Ya. Kagan, N. F. Gamaleya Institute of Epidemiology and Microbiology, AMN, USSR, which appeared in the Journal of the USSR Academy of Medical Science, 15.11:25-38, 1960. Translation performed by Sp/6 Charles T. Ostertag Jr.]

L-forms of bacteria were first discovered in 1935 by Klieneberger who isolated them from a culture of Streptobacillus moniliformis and named them the L_1-form in honor of the Lister Institute. In spite of the passage of 25 years since the discovery of L-forms of bacteria, their biological nature, association with other forms of mutability, importance in the evolution of microorganisms, and their role in infectious pathology are items of intensive study and dispute up to the present time.

In her first works Klieneberger clarified her proposed hypothesis, which proposed the L-form in a bacterial culture of Streptobacillus moniliformis was to be considered as one of the symbionts of two microorganisms (bacterial and peripneumonic), supposedly lacking in the mutual exchange products for their own normal development. In subsequent experimental work by Dienes et al., Klieneberger's hypothesis was disproved when the authors succeeded in proving that the L-form is not a symbiont but a markedly changed variant of a culture of Streptobacillus moniliformis.

The bacterial origin of the L-form was corroborated in the works of Heilman et al. and then even Klieneberger-Nobel, who having verified the results of the experimental works of Dienes et al., renounced her own hypothesis of symbiosis.

The biological nature of L-forms is discussed even in the present day. A. A. Imshenetskiy identifies the L-forms of bacteria with "pettenkoferia" and other forms of mutation arising in response to unfavorable influences. [The term "pettenkoferia" was used by Kuhn to designate the big "aberrent forms" which arose as the cells of a parasite of the bacteria - Kuhn, P. and Sternberg, K., 1931 Ueber Bakterien und Pettenkoferien. Zentr. Bakt., I, Orig. 121, 113-161. Cited from Bacterial Reviews 14-15, 1950-51, p 93.] In his

opinion the L-forms should be regarded as degenerate, cytopathological forms, destined for death. Other investigators (M. A. Peshkov; V. D. Timakov; G. Ya. Kagan; B. Ya. El'bert; Klieneberger-Nobel; Minsk; Tulasne and Brisou, and others) reject such an interpretation of the L-form, considering them as forms, highly stable in relation to the factors which caused their formation and emerging as a result of the adaptation of bacteria to changed conditions of existence.

An analysis of the factual material presented in this article makes it possible to reveal the biological nature of the L-forms of bacteria and differentiate them from other forms of mutation.

Variants, formed under the influence of various interactions, are related to the L-forms of bacteria. Several amino acids are the most active L-transforming agents, for example, glycine, carboxylmethoxylamine, dl methionine, l - phenylalanine and others; antibiotics - penicillin, for some species of bacteria - streptomycin, bacteriophages, immune serum and complement.

Transformation of bacteria into L-forms depends on the species of bacteria, status of the population, selection of transforming agent and the conditions of cultivation. Certain species of bacteria, for example, Streptobacillus moniliformis, Pr. vulgaris, Salmonella, form L-forms more often than others, for example, C. diphtheriae, or some pathogenic cocci (streptococci Together with the factors causing the formation of L-forms in various species of bacteria (penicillin), factors are known, the L-transforming action of which is strictly differential depending on the species; thus, for example, streptomycin, to which the L-forms of many species of bacteria are sensitive, is the L-transforming agent only for tubercular mycobacteria (Huertas).

The conditions of cultivation, contributing to the transformation of bacteria into L-forms, are not the same for different species of bacteria. General conditions, regardless of species origin, are the need for normal horse serum or its substitutes (polyvinylpyrrolidone or vitamins of group B), which destroys the inhibitors to the growth of the L-form which are available in common nutrient media, a semisolid or semiliquid consistency of the media, and an increase of their hypertonicity due to an augmentation of the osmotic regulators. Included as substances which stabilize the actions of transforming agents are saccharose, $MgSO_4$, KH_2PO_4 and Na_2HPO_4, NaCl, KCl, $CaCl_2$, $LiCl_2$, and others.

The presence of similar regulators in the medium is quite necessary for obtaining and cultivating L-forms of coccal species of bacteria; their presence is optional for Gram negative rod-shaped bacteria and is not required at all for such Gram positive rod-shaped bacteria as C. diphtheriae, which in the presence of increased concentrations of NaCl, KCl, $CaCl_2$ and phosphate

salts, rapidly disintegrates and perishes under the influence of the transforming agent, not having changed into L-forms (G. Ya. Kagan and V. T. Savenkova).

A detailed analysis of experimental data received by us in the laboratory during the study of the L-forms of various pathogenic species of bacteria (V. D. Timakov, G. Ya. Kagan, V. S. Levashev, S. G. Komm; V. D. Timakov, G. Ya. Kagan; G. Ya Kagan and V. S. Levashev; G. Ya Kagan and V. T. Savenkova; G. Ya. Kagan and Z. A. Pesina: V. S. Levashev; S. V. Prozorovskiy) and the materials of other investigators (M. A. Pesh[illegible]; [illegible] [illegible]ershina; Klieneberger-Nobel; Dienes; Tulasne; Minsk; Taubeneck and many others) show with certainty that the L-forms of bacteria have a number of biological peculiarities, differentiating them from other forms of mutability.

L-forms of bacteria grow on semisolid (1.3% agar) and semiliquid (0.3% agar) nutrient media in the form of characteristic L-colonies with a delicate, transparent, broken periphery of a slimy consistency and a rather dry, thickened, easily pigmented, center growing into the depth of the medium. Based on the form of the colonies, the L-forms of various species of bacteria are the same. The dimensions of the colonies vary from 0.1-0.5 mm in diameter up to 2-3 mm depending on the species of the bacteria and the cultivation medium. In many species of bacteria, for example R. vulgaris, Pr. mirabilis, E. Coli, V. Cholerae and Streptococcus haemolyticus, the L-colonies are differentiated into two types, these are the so-called "3B" and "3A" colonies (fig 1).

The "3B" colonies have larger dimensions than "3A" colonies. During prolonged storage they acquire a yellowish-brown color owing to the production of the pigment - flavin. They adsorb the corresponding bacteriophages well and are lysed by them. They retain phage sensitive receptors and are agglutinated by the serum of the original species of bacteria. They are subinoculated in subcultures on media containing the transforming agent and easily reverse into the original culture of bacteria with the elimination of the transforming agent from the composition of the medium. "3B" colonies are something like an intermediate phase of L-transformation.

Type "3A" L-colonies form cultures of stabilized L-forms and have smaller dimensions than "3B" colonies. They do not adsorb the corresponding bacteriophages since they lose the phage sensitive receptors. Often they are not agglutinated by the serum of the original species of bacteria. They are subinoculated in subcultures independent of the content of transforming agent in the medium. For the most part they do not revert into bacterial forms. "3A" colonies represent the concluding phase of L-transformation. According to microscopic structure "3A" and "3B" L-colonies are completely the same.

Individual microstructural elements, components of the L-colony, represent extremely peculiar analogs of cells, and, regardless of the sharply expressed polymorphism, they are easily differentiated from any other forms of mutability. In the composition of L-colonies there are found spherical, bladder-like, sometimes pear shaped and branching forms from 1 up to 20 microns in size and of a different optical density, formations of an uncertain, constantly changing configuration which are dense and transparent and distinguished by a different refraction. There is a multitude of different vacuolozed forms containing one or several vacuoles. In some species of bacteria - Pr. Vulgaris, Ps. Fluorescens, very long and thin flagellates are attached to the spherical bodies (Kohler, Huertas). Submicroscopic filterable forms with a size from 100 up to 500 millimicrons are also constitutent microstructural elements of L-colonies. They are located within the spherical, vacuolized and other bodies in the form of inclusions, and also outside of these formations in the form of loosely lying masses, forming the center of the L-colony growing into the agar. The submicroscopic, filterable forms have a spherical ellipsoidal form, are characterized by Brownian movement (Huertas), pass through bacterial filters and are precipitated out during ultra centrifugation (Klieneberger-Nobel, Tulasne, V. S. Levashev, 1960). All the microstructural elements of L-forms are fixed poorly and are not stained by the Gram method. Together with the spherical bodies which are stained blue with the Giemsa stain and which contain red inclusions, there are bodies, always filled with nuclear substance, surrounded only by a narrow stria of basophilic cytoplasm (M. A. Peshkov, Nermut). As a result of the disruption of the cytoplasmatic and intensive nuclear division, the cytoplasm of large spherical and other bodies is filled with submicroscopic granules of a nuclear origin (Tulasne, Nermut), as a result of which in the L-forms of bacteria there takes place a sharp reduction of the RNA/DNA coefficient, that is an increase in DNA. Separate L-elements of the "3A" type are distinguished from "3B" by a change of the chemical composition of the cell walls, by the loss of a-E diaminopimelic acid (Taubeneck; Lederberg and St. Clair).

The morphogenesis of L-forms of bacteria is distinguished by the diversity of their development. In the initial phase of the transformation of bacterial cells into an L-form a disruption is noted in the function of division with the preservation of growth, as a consequence of which bodies are formed which are diverse in form and which grow to gigantic sizes. Thus in representatives of Gram negative rod-shaped bacteria most of all in the initial phase of morphogenesis, spherical bodies (fig 2) are formed; in some Gram positive rod-shaped bacteria, for example C. diphtheriae, long flagellates and bladder like forms (fig 3); in hemolytic streptococci along with the formation of spherical bodies there takes place, due to the destruction of the cell wall, the liberation of structureless granular masses of cellular contents. Morphogenesis of L-forms is concluded with the complete disappearance of bacterial cells of the initial species, which are replaced by the microstructural elements of L-forms; L-colonies are formed which are no different in the external

appearance and morphology of the individual microstructural elements in various species of bacteria (fig 4, 5, 6, 7).

A characteristic peculiarity of the L-forms of bacteria are the diverse types of their multiplication. Individual microstructural elements of L-forms multiply by simple division, division in various directions, budding (fig 8), and the formation of submicroscopic granular forms which are capable of growing into large spherical bodies which break up again into submicroscopic granules.

The varied nature of multiplication and the reproducing capabilities of all microstructures of L-forms: Spheres, vacuolized bodies without a specific configuration and submicroscopic forms, determine the capability for the prolonged preservation of the cultures in the L-form and ability to pass into subcultures.

The prolonged subculturing of L-forms under conditions where a transforming agent is present may be accompanied by 1) temporary stabilization, that is the capability to be subinoculated in the L-form in media containing an L-transforming agent, 2) permanent stabilization, that is the capability to be subinoculated in an L-form independent of the content of transforming agent in the medium, and 3) by breaking up into submicroscopic filterable forms passivating in subcultures.

The stabilization of cultures in the L-form has been noted at the present time in many species of bacteria: Pr. vulgaris (Tulasne, V. S. Levashev), V. cholerae (Minsk, Lavillaurix), S. typhosa (G. Ya. Kagan and V. S. Levashev), C. diphtheriae (G. Ya. Kagan and V. T. Savenkova), Staph. aureus (S. V. Prozorovskiy), and Streptoc. haemolyticus (G. Ya. Kagan and V. S. Levashev).

A special feature of the chemistry of the L-form is the concentration of synthetic processes, directed to the preservation of the nuclear substance of the cell (Tulasne, M. A. Peshkov, Nermut); as we already pointed out a disruption of cytoplasmic division occurs with the retention of nuclear division. This process involves a change in the composition of nucleic acid due to an increase of the number of submicroscopic elements of the L-forms, consisting mainly of DNA, which are contained in all the microstructures of L-colonies (intracellular inclusions in large bodies and vacuolized forms, extracellular structures forming the center of L-colonies, grain accumulations and structureless masses, almost without exception consisting of submicroscopic granules). In the process of L-transformation, polysaccharide, protein and water exchange varies. Together with the loss or inhibition of some fermentation systems, inherent to the original species of bacteria, for example in the L-forms of Proteus vulgaris, S. typhosa or Staph. aureus (V. S. Levashev; V. D. Timakov, G. Ya. Kagan and V. S. Levashev; S. V. Prozorovskiy), there is observed the acquisition of new systems, not characteristic to the original

culture, for example the ability to ferment maltose (V. S. Levashev) and urine in the L-form of Proteus vulgaris (Minsk), to synthesize cholesterol and flavin or to promote anaerobic glycolysis (Minsk; Dienes). Changes to the chemical structure and metabolism consequently lead to an increase in the degree of stability to factors which cuased the formation of the L-form, for example to penicillin (V. S. Levashev, S. V. Prozorovskiy), carboxylmethoxylamine, glycine (Dienes), or streptomycin (Huertas) and also in changes of antigenic structure. The latter, maintaining species specificity and general antigenic groupings with the initial cultures, changes. These changes are expressed in the acquisition during the process of L-transformation of specific antigenic components (Tulasne, Weinberger, Madoff and Dienes; V. S. Levashev; V. D. Timakov, G. Ya. Kagan and V. S. Levashev; G. Ya Kagan and Ye. I. Koptelova, 1960).

A particular place in the problem of L-forms of bacteria belongs to the investigation connected with the study of the L-forms' capability to reverse into bacterial cultures. It is difficult to overestimate the value of this most important biological peculiarity of L-forms for biology and especially for medicine. Reversion of L-forms into bacterial cultures takes place sometimes without any kind of artificial influences on the population; such an origin of reversion may be conditionally called "spontaneous", it is most often observed in freshly isolated cultures of L-forms which have not succeeded in becoming stabilized (Dienes; M. A. Peshkov; G. Ya. Kagan and V. S. Levashev; S. V. Prozorovskiy). Artificially produced reversion is observed most of all when the L-transforming agent is eliminated from the nutrient medium.

Morphological investigation of the process of reversion (V. D. Timakov, G. Ya. Kagan, V. S. Levashev, S. G. Komm; V. D. Timakov and G. Ya. Kagan; M. A. Peshkov; Tulasne; Kuhl and Liebermeister; Hopken and Bartman) testifies to the multiple nature of the morphogenesis of reversion, which is approximately the same in L-forms of various gram-negative species of bacteria. Reversion takes place either by means of an elongation of large spherical bodies and the formation of beaker-shaped, fusiform, irregularly shaped or long winding cells, segmented into bacillary forms or by means of segmentation of large bodies and their disintegration into curved bacterial forms. The formation has also been described of transitional branching forms from vacuoles which bud off bacilli. Pictures 9-11 depict the film documentation of the process of reversion of L-forms of S. typhosa followed by us in a Fonbrune oil chamber by using time lapse motion picture filming. Reverse forms are mainly spherical bodies which most often by the end of the first 24 hours are becoming elongated, converting into cells that are irregularly formed, or oblong or beaker-shaped; part of the cells die, the others become still more elongated, transforming in the course of 30-40 hours into gigantic winding long forms (fig 10) which are segmented and which bud off normal bacterial cells (fig 11).

The reversion of L-forms of coccal species has been studied little. A description of this process is encountered only in single works (S. V. Prozorovskiy; G. Ya. Kagan and V. S. Levashev). According to data of S. V. Prozorovskiy, the first phase of morphogenesis of reversion of L-forms of Staphylococcus aureus is the gradual vacuolization of a large portion of the structural elements of the L-colonies. Almost without exception, the latter consist of large and medium size vacuoles, gradually filling up with large grains which are very close in external appearance to fine cocci. This process is concluded with the formation of coccal forms. The periods of vacuole regeneration of L-colonies and the formation of granular forms vary, they may be computed by days, weeks or months depending on the length of the reversion process in the individual strains of the L-form.

The above presented results of morphological investigations of the reversion process testify to its complexity and species variations. All structural elements of L-forms (winding forms, spherical and vacuolized bodies, submicroscopic structures) possess a regenerative function capable of the reversion of bacterial cells. The regenerative function of all the microstructures of L-forms and the multiple nature of reversion (segmentation of large bodies into bacillary forms, conversion into beaker shaped, fusiform, winding and other forms which bud off or break up into bacilli) in various bacterial species testifies to the latitude of the adaptive functions of bacteria, which in the L-form preserve the capability for many methods of reversion.

The reversion of L-forms depends on two conditions: On the conditions of the media in which it is taking place and on the state of the population of L-forms, that is the level of stabilization of the newly acquired features of L-cultures. The higher the level of stabilization, the more difficult and complicated the process of reversion into bacterial forms (data by G. Ya. Kagan and V. S. Levashev during the study of reversion of L-forms of S. typhosa and Streptoc. haemolyticus, Minsk - Cholera vibrio; Tulasne - Proteus vulgaris, S. V. Prozorovskiy - Staphylococcus aureus).

The difficulty of reversion of stabilized L-cultures is a classic example of the stability of features acquired in the process of adaptation, since the longer the process of adaptation the more complicated and difficult the return to the initial form. This is also supported by the nature of regeneration of features of the initial cultures in the process of L-form reversion. Very noteworthy in this respect is the fact of the complete regeneration of all the biological features of the initial cultures including fermentative, antigenic, pathogenic and sensitivity to the L-transforming agent in L-cultures of the first passages, for example in S. typhosa (G. Ya Kagan and V. S. Levashev), that is with less stabilization of "L-features".

In studying the biological properties of bacterial cultures reversed from stabilized L-forms of the typhoid fever causative agent, hemolytic streptococcus and Staphylococcus aureus, it was established that complete regeneration of the features of the initial species didn't take place (G. Ya. Kagan and V. S. Levashev; S. V. Prozorovskiy).

The results are also interesting of other experiments carried out by u in the laboratory (G. Ya. Kagan and V. S. Levashev, 1958). These testify to the fact that in cultures reversed from L-forms there is noted an expressed capability toward L-transformation. They easily and rapidly produce formatio of L-colonies when inoculated on media containing penicillin. In this it mus be noted that alternate existence of cultures in the L- and reversed bacterial form leads to a greater and greater change of biological features of the latter, to atypical fermentative and antigenic properties (fig 12). As is apparent from this diagram, in contrast to the reverted cultures obtained from the first transformed L-forms, completely reverting to the biological features of the initial type, cultures obtained upon reversion of L-form which have been transformed two and three times from reverted cultures, are atypical and do not regain the biological features of the initial species of bacteria.

In considering the nature of L-forms of bacteria, one cannot stop at their connection with heteromorphic variants, protoplasts, filterable forms, and also organisms similar to pleuropneumonia.

It was established by the comprehensive investigators, N. F. Gamaleya, M. P. Pokrovskiy, M. A. Peshkov, A. A. Imshenetskiy, and others that under the influence of unfavorable media factors (cultivation at high and low temperatures on media containing increased concentrations of carbohydrates or the salts of Mg, Na, Li, and others) there sometimes are formed giant bacteria forms growing in the shape of spheres, fusiform shapes and filaments which N. F. Gamaleya designated as "heteromorphic". These forms are not connected with degenerative and involution forms, their formation in cultures is evidenc by profound and specific changes in the nuclear apparatus of the bacteria (N. Gamaleya). M. A. Peshkov's point of view is close to N. G. Gamaleya"s. Peshkov's experimental works made it possible to classify heteromorphic growth into forms of natural (Achromobacter epsteini) and forced polymorphism.

A. A. Imshenetskiy has a completely different interpretation of the nature of heteromorphic growth. While studying the nature of the influence of concentrated solutions of several salts on individual representatives of Bacteriaceae and Bacilaceae, the author observed the appearance of gigantic sphere like forms which were exposed to "vacuole degeneration" and disintegration into elements consisting of nuclear substances. The ultimate fate of these "reaction forms" is concluded either with destruction or a return to the initial bacterial forms. The author considers heteromorphic growth as the effect of the degeneration of bacteria.

An analysis of experimental data obtained by us in the laboratory while studying heteromorphic variants of staphylococcus, gonococcus and C. diphtheriae (V. S. Prozorovskiy; G. Ya. Kagan and A. A. Pesina; G. Ya. Kagan and V. T. Savenkova) and the results of the works of M. A. Peshkov and other investigators do not allow that heteromorphic variants be considered as cytopathological forms.

Heteromorphism from out point of view is one of the forms of the biological reactions of bacteria enabling their survival and preservation under conditions of the temporary unfavorable influence of some physical, chemical or biological agents.

Heteromorphism is a temporary initial form of adaptation of bacteria, and is capable of converting into more perfect and constant forms of adaptation. A comparison of the biological features of heteromorphic variants with L-forms of bacteria testifies to the presence of a similarity and distinctions between them.

Heteromorphic variants compare with L-forms in a certain similarity of morphological structures and the formation of gigantic sphere-like forms which are vacuolized and contain granules of a nucleated nature. In contrast to L-forms they originate in response to the short-termed action of small doses of destructively acting factors and are capable of prolonged cultivation in subcultures; a cessation of the action leads to a rapid reversion of bacterial forms of the initial species.

Observations of the formation process of L-forms by Staphylococcus aureus (S. V. Prozorovskiy) and gonococcus (G. Ya. Kagan and Z. A. Pesina) showed that, depending on the concentration of penicillin and salt stabilizers in the medium, there is observed a differentiating reaction of bacterial cultures, the formation of heteromorphic variants in low concentrations of the specified agents and the formation of L-forms at increased concentrations and a lengthier effect. When heteromorphic variants of Staphylococcus aureus are passaged on media with increasing concentrations of penicillin it is possible to obtain the growth of stabilized L-cultures (S. V. Prozorovskiy)

The prolonged passaging of heteromorphic variants of C. diphtheriae on media with increasing concentrations of an L-transforming agent may be accompanied by the conversion of part of the strains being passaged into L-forms (G. Y. Kagan and V. T. Savenkova).

Thus the results of a comparative study of some biological features of heteromorphic and L-forms testifies to the fact that heteromorphic forms may be seen as one of the initial transitional phases of development of L-forms of bacteria.

In the process of formation of L-forms regardless of the fact that they were formed as a result of the original reaction of the bacteria on cultivation under conditions of penicillin - serum - salt media or by means of the gradual cultivation of heteromorphic forms on media with increased concentrations of penicillin, the stability of the L-forms to penicillin increases 10,000 times in comparison with the original culture. Along with this, reversion isn't observed on media with smaller concentrations of penicillin.

The resistance to penicillin of the originally obtained heteromorphic variants of staphylococcus increases 10-50 times; their gradual passaging, without converting into L-forms, on media with increased concentrations of antibiotics leads to an increase of resistance to penicillin, but never reaches the degree of stability of L-forms. Contrary to the L-forms, as a measure of increasing the stability of heteromorphic variants, their cultivation on media with lower concentrations invariably is accompanied by reversion. With the exclusion of penicillin from the make-up of the medium, the reversion of heteromorphic forms into coccal forms takes place in 4-5 days, L-forms considerably slower - from 1 to 5 months depending on the degree of stabilization of the culture in the L-form (S. V. Prozorovskiy).

Cultures which reverted from heteromorphic forms completely recover the properties of the initial cultures, including plasma coagulating, necrotoxic and virulent properties. Complete recovery of the properties of initial staphylococcal cultures in the process of their reversion from L-forms were not observed. It should be noted here that the degree of recovery of features is in direct connection with the level of stabilization of the culture in the L-form (S. V. Prozorovskiy).

Thus heteromorphic forms, which differ from L-forms by instability and lability of features newly acquired by them, easily reverse into the original species upon restoration of the initial conditions of cultivation and perish under the prolonged action of the factor which caused their formation in the cultures, or are converted under appropriated conditions into an L-form which is new according to its biological quality. Therefore heteromorphic forms should be considered as a temporary, fluctuating form of mutability of microorganisms, which is not accompanied by stabilized changes which are passed on through inheritance, but only temporarily an increased adaptability of the microorganism for a relatively short time to changing conditions of the medium of inhabitation.

A comparison of the biological peculiarities of heteromorphic variants with forms of an unfinished L- or M- cycle (Tulasne and Lavillaureix; Tulasne and Brisou) testifies to their complete identity. They are similar according to conditions of formation, morphological properties, the ability for a rapid and complete reversion of bacterial forms upon the elimination of the action of the transforming agent, and also they perish under a prolonging or strengthening of its action.

A prolonged passaging of these forms on media, containing the transforming factor may sometimes end with their conversion into L-forms.

The initial phase of the formation of L-forms of bacteria may be the formation of bacterial protoplasts. They, like L-forms of bacteria, are formed due to the actions which are blocking the synthesis of the dense cell walls (Weibull; Park and Strominger). Thus the addition of lysozyme to a culture of Micrococcus lysodekticus or B. megatherium under conditions of increased osmotic pressure is accompanied by depolymerization and complete depletion of cell walls and the formation of spherical cells - lysozyme protoplasts; during this, other vitally important cell functions are not affected (Salton; Weibull).

The morphological similarity of penicillin protoplasts and L-forms is manifested in that the spherical dense bodies are not only a morphological expression of protoplasts but also a constituent component of L-forms. Protoplasts have the form of spherical bodies which increase in size during prolonged incubation. Often "moon-like" vacuoles are formed in them (Laderberg and St. Clair) and they take on the shape of a half-moon, made up of a dense substance bordering on the side of a fully transparent vacuole. Within the vacuoles and dense spherical bodies, upon aging of the protoplasts, a great number of dark granules are formed, for example in E. coli (Laderberg and St. Clair), Pr. vulgaris, S. typhosa, and Strept. haemolyticus (G. Ya. Kagan and B. S. Levashev, 1960). The protoplasts, just like the L-forms, do not take the Gram stain and are distinguished by a certain osmotic friability, caused by the nature of the effect of penicillin on the cell wall. Possessing the capability of assimilation, the protoplasts produce division forms though they are not capable of subsequent regeneration, do not reproduce and are not transferred into subcultures (Weibull; Klieneberger-Nobel).

Morphologically, L forms of bacteria are differentiated from protoplasts by a diversity of microstructures described above which are not detected in protoplasts. The sphere-shaped bodies of L-forms, in contrast to protoplasts, reach very large dimensions and possess the ability to multiply and form cultures, for example a culture of L-forms of V. cholerae (Lavillaureix), consisting mainly of sphere shaped bodies, and culturing for a long time in sub passages. The progressive passaging of protoplasts on solid nutrient media which contain an L-transforming agent may be completed with the formation of L-cultures of the "3B" and "3A" types; L-forms have been obtained from protoplasts of E. coli, Pr. mirobilis, and S. Typhosa (Weibull, Lederberg and St. Clair; Klieneberger-Nobel; Bohme and Taubenek; Landman, Altenberg and Ginoza; G. Ya. Kagan and V. Levashev, 1959). Thus, protoplasts, just as the heteromorphic variants, appear as the initial phase of development of the L-forms of bacteria.

As we already pointed out, in the process of the formation of L-variants granular submicroscopic elements are formed. They are found inside of the larger bodies and outside of them. These elements pass through bacterial filters. They form the center of L-colonies and are capable of reproduction and reversion into bacterial forms. The problem of the connection of these filterable elements of L-forms and filterable forms of bacteria is the object of consideration in contemporary bacteriological literature (M. A. Peshkov; Huertas; Klieneberger-Nobel).

A comparison of some biological features of filterable elements of the L-forms and filterable forms of bacteria testify to their biological nearness. Filterable forms of bacteria are morphologically analogous with filterable elements of L-forms. They have a spherical or oval form with a diameter from 0.1 to 0.3 microns (B. N. Il'yashenko; V. D. Timakov, G. Ya. Kagan, Ye. I. Koptelova, N. N. Solov'yev; V. S. Levashev, 1960, Juhasz, Lovas and Egyessy; Huertas). They pass through bacterial filters and precipitate out during ultra centrifugation (Klieneberger-Nobel; Tulasne; Mandel and Terranova; V. S. Levashev, 1960). Filterable forms are made up mainly of DNA, but also contain RNA and other proteins (Juhasz; V. S. Levashev, 1960). Thus even in chemical composition they are also close to filterable elements of L-forms and are distinguished from them only by quantity. It is known that during the disintegration of unchanged or mildly changed cells the number of filterable forms is not great; in heteromorphic variants they may be detected in the form of a considerable number of inclusions of a nucleate nature contained in a giant changed bacterial cell; they are observed in maximum numbers in L-forms of bacteria. A sharp increase of their number during the formation of L-forms of bacteria is conclusively shown in the experiments of Tulasne, Nermut and other authors.

Analysis and other biological features testify to the biological community of filterable forms and filterable elements of L-forms of bacteria. Thus it is well known that when filtrates of L-forms are inoculated on the appropriate media it is possible to obtain the growth of L-colonies. V. S. Levashev (1960), when inoculating a filtrate of an aging culture of Pr. vulgaris on a medium favorable for the growth of L-forms of the stated causative agent, was able to obta.. the growth of single colonies of L-forms.

Filterable elements of L-forms as well as filterable forms regenerated into bacterial cultures slowly and with difficulty. These cultures are differentiated by a retarded and sparse growth and by lowered fermentative and antigenic properties and are considerabley different from the original bacterial cultures. (V. D. Timakov, G. Ya. Kagan, Ye. I. Koptelova, N. N. Solov'yev; G. Ya. Kagan and V. S. Levashev).

In summarizing the comparative data, characterizing filterable elements of L-forms and filterable forms of bacteria, their definite biological association must be noted. They are elementary reproducing and regenerating units of bacterial cells, the biological purpose of which is the preservation of the life of the bacteria under changed conditions of existence.

Morphologically and physiologically close to the L-forms of bacteria is the group of so-called pleuropneumonia-like organisms (PPLO). These microorganisms are different from bacteria, rickettsiae and viruses. They, just like the L-forms of bacteria, grow on cell-less media which contain additional substances: Normal serum, ascitic liquids, cholesterol, mucin, DNA, and others. PPLO colonies in external appearance, size and microscopic structure are close to stabilized L-forms - colonies of the "3A" type. The dimensions of the colonies vary from 250 up to 750 microns and higher. The periphery, as in L-forms, consists mainly of diverse, sphere-like and irregularly formed bodies and columnar structures; the center, which is growing into the medium, consists of submicroscopic filterable granules and very fine filaments and is easily pigmented (Poetschke, Edward, Minck, Morton).

The various representatives of this group of microorganisms are morphologically similar and can hardly be distinguished from L-forms of bacteria. They are made up of microstructural elements of various size and form, sphere like bodies, annular vacuolized and filamentous forms, and filterable granules. Myceliel structures are encountered more often in PPLO cultures than in L-forms (Freundt); filterable elements are distinguished by somewhat smaller sizes - 100-150 millimicrons (Edward, Klieneberger-Nobel). The filtration end point through Gradocol membrane filters for PPLO = 0.25 microns and for L-forms - 0.45 microns. The differences encountered in the details of microscopic structure and the sizes of individual microstructural elements are so insignificant that they cannot be considered as criteria for the differentiation of individual species of PPLO from each other and all of this group from the L-forms of bacteria.

Just as L-forms of bacteria, PPLO are differentiated by the multiple nature of reproduction, which takes place by means of segmentation of filamentous forms (Orskov, Dienes, Freundt), division and budding, and also by the intracellular development of submicroscopic elements formed in the sphere like bodies and released during their disintegration (Klieneberger and Smiles). Electron microscope research (Klieneberger-Nobel, Norton) testifies to the fact that in many representatives of PPLO as well as in stabilized "3A" type L-forms, the cell wall is lacking.

Based on the nature of growth and nutritive requirements, PPLO forms are close to L-forms of bacteria (Minck; Edward). Several species of PPLOs

are distinguished by the fact that they are lacking in supplementary nutrition factors, for example yeast extract and especially cholesterol. Cholesterol, which is a vitally necessary growth factor of several PPLO forms, is not needed for the growth of L-forms, which are capable of synthesizing it (Morton). In contrast to L-forms, PPLO forms grow well on appropriate nutrient media immediately in the first inoculations. L-forms grow poorly in the first generations, going through a stage of adaptation to the nutrient medium. As the number of passages increases they grow more intensively.

The problem concerning the identity of PPLO forms and stabilized L-forms of bacteria has been the object of dispute up to the present time (Edward, Klieneberger-Nobel, Minck, Morton). The community of the biological features of these groups of microorganisms makes it possible to speak about the community of their origin. In spite of the differences of some details of structure and metabolism, they are basically identical not only according to morphology, nature of growth and peculiarities of multiplication, but also by other features.

PPLO forms, like the stabilized L-forms, lose the fundamental component of the cell wall - diaminopimelic acid (Kandler and Lehender). They are distinguished by osmotic friability, sensitivity to streptomycin, aureomycin and terramycin, and a high stability to penicillin, thallium acetate and other L-transforming agents (Dienes, Pulvertaft).

The basic distinction betwen PPLO forms and L-forms of bacteria is their lack of reversion into bacterial cultures. However even this difference has only a relative significance. Thus at the present time it is well known that stabilized L-forms of many species of bacteria persistently lose the capability of reversion into the original species of bacteria. These stabilized L-forms are practically no different from PPLO forms. The feasibility has also been established of the reversion of several species of PPLO forms into bacterial forms. For example in the experiments of Moustardier, Brisou and Perry, reversion was obtained into bacterial cultures of Streptoc. faecalis, B. faecalis alcaligenes and Pr. mirabilis of 4 strains of PPLO isolated from patients with non-gonococcal urethritis. Wittler was also able to observe reversion of a strain of PPLO in a tissue culture into Corynebacterium.

Thus the community of the basic morphological and physiological features of PPLO forms and stabilized L-forms of bacteria testify to the fact that PPLO forms originate from L-forms, which appear as though they were a transitional phase in the course of the evolutionary development of PPLO forms from bacteria.

Recognition of the biological community of PPLO forms and L-forms of

bacteria is reflected in the classification system suggested by Tulasne and Brisou in accordance to which the basic criterium for the differentiation of PPLO forms and L-forms is the fact of the proven bacterial origin of the latter. In connection with this all the representatives of PPLO forms are regarded in the order Pleuropneumonialis, close to the family Borrelomycetaceae Parasitaceae and the genus Asterococcus (Borel). This order contains only one family Pleuropneumonieaceae and one genus Pleuropneumonia and is divided into the individual species: Pl. bovis, Pl. agalactiae. Pl. artitidis muris, Pl. cerebri muris, Pl. aviae, etc.

Acceptance of the biological community of PPLO forms and L-forms of bacteria into the group Bacteropneumoniales has been abbreviated Bp. In this group are treated the L-forms of all the bacterial species from which they were obtained, for example: Bp. Proteus, Bp. Salmonella, Bp. Escherichia, etc.

The above presented results of the experimental investigations of the nature of L-forms of bacteria and their relation with other similar forms of mutation permits the classification of these forms in relation to the system presented below (fig 13).

According to this arrangement, all forms of mutation, emerging in response to the influence of various physical, chemical and biological agents, may be divided into the following three groups: 1. Variants with temporarily altered features (modified forms of mutation), 2. Variants with hereditarily fixed altered features, 3. Degenerative-involution forms.

Heteromorphic or M-variants and subcellular units: Protoplasts, non-stabilized L-forms and submicroscopic filterable forms are regarded as variants with temporarily altered features. Variants with temporarily altered features may reverse into the initial species of bacteria and transform into stabilized L-forms, these are temporary adjusted forms which ensure the survival of the bacteria in specific short-termed changes in the conditions of the medium.

Stabilized L-forms, pleuropneumonia like organisms or OTPP (PPLO or OTPP -- organisms of the peripneumonia type) and submicroscopic forms are regarded as variants with hereditarily fixed features.

Stabilized L forms originate from the above described variants with temporarily changed features; in the course of their subsequent development and breeding they may produce the formation of new species of PPLO forms and also disintegrate into submicroscopic subcultured forms which preserve their vitality. Variants with heretically fixed altered features, including stabilized L-forms, are the most ideal form of adaptation of bacteria to changed conditions of existence, principally distinct from degenerative forms which have not developed adaptive arrangements and on the power of this are non viable.

PPLO is an example of the formation of new species during the process of the evolutionary development of bacteria. The basic driving forces contributing to their origin in the course of evolution is the formation of adap ive mechanisms and breeding which culminates in the formation of new species of PPLO.

Figure captions (between pages 28 and 29)

1. "3A" and "3B" type colonies of haemolytic streptococci.

2. Initial phase of the formation of L-forms.

3. Initial phase in the formation of L-forms in Str. haemolyticus. 1500x.

4. Microstructures of L-colonies of S. typhosa. 1500x.

5. Microstructures of L-colonies of C. diphtheriae. 1500x.

6. Microstructures of L-colonies of Streptococcus haemolyticus. 1500x.

7. Microstructures of L-colonies of Staphylococcus aureus. 1500x.

8. Multiplication of L-forms of khurpoz* by budding. See explanation in the text. 1500x. [*The word Khurpoz is unknown. Presumably it is meant to refer to a species of microorganisms, which can not be established. The explanation in the text doesn't clear anything up.]

9. Goblet like cells of a form of reversion of L-forms of S. typhosa. 1500x.

10. Cells of irregular forms and gigantic twisting forms of reversion of L-forms of S. typhosa. 1500x.

11. Lengthy forms, segmenting and budding off normal bacterial cells of the L-form. 1500x.

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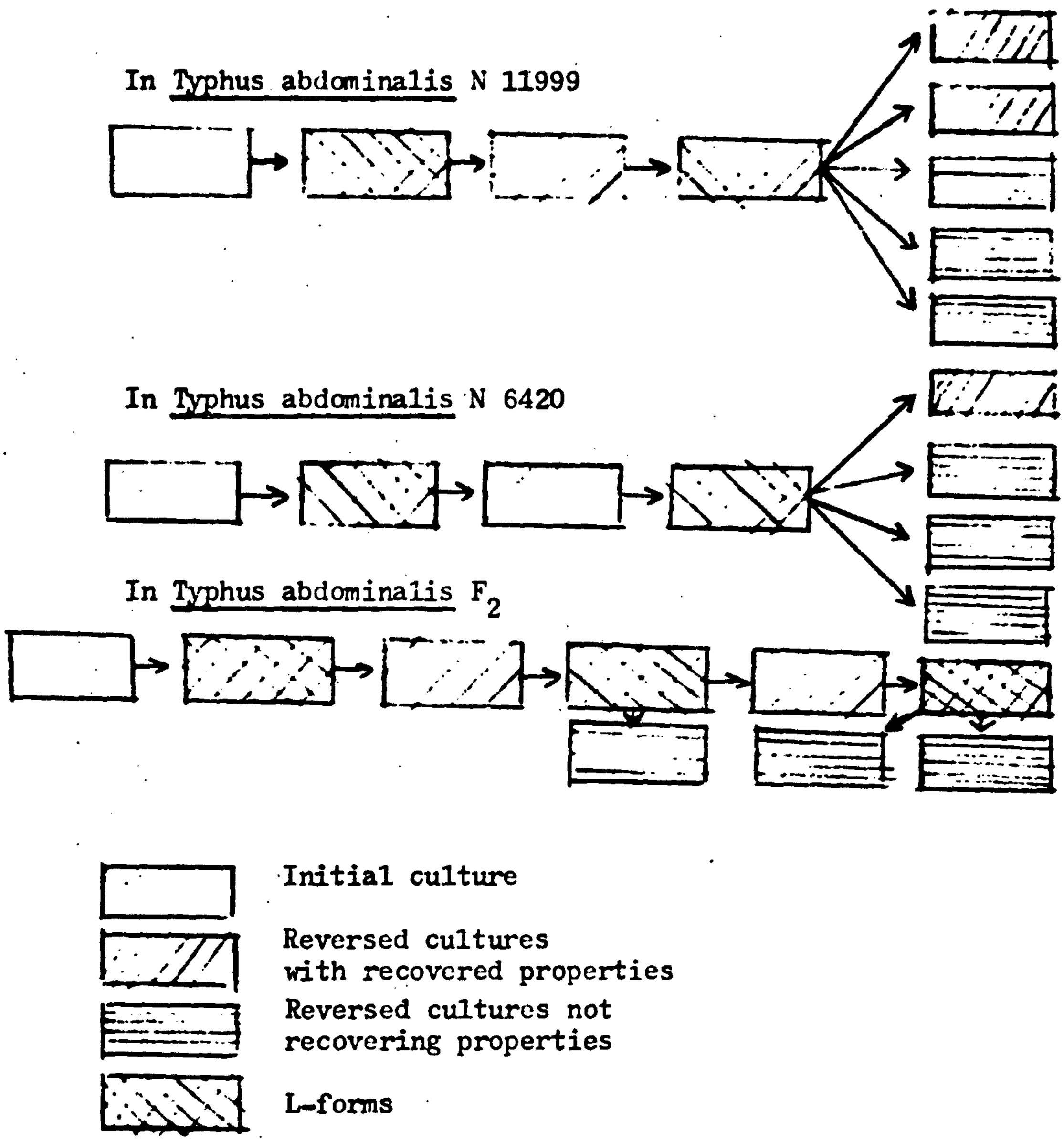

Fig. 12. Reversion of features depending on alternate residence of the cultures in the L and bacterial form.

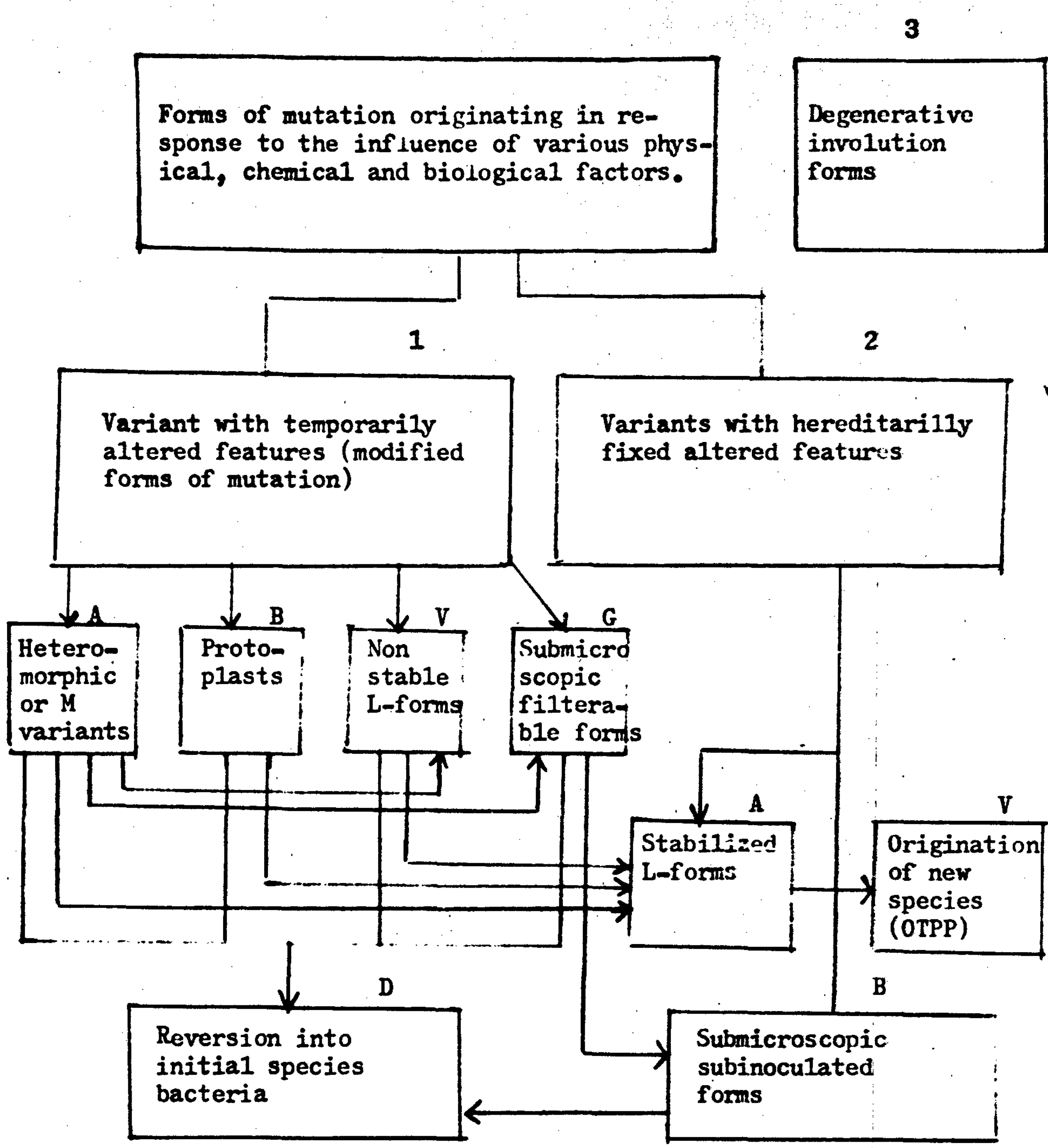

Figure 13. Biological reactions of bacteria in response to the influence of physical, chemical, and biological factors.